Sperm chromosomal aneuploidy and DNA integrity of infertile men with anejaculation

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Abstract

Purpose To explore sperm chromosomal aneuploidy, sperm membrane and DNA integrity in infertile patients with anejaculation.

Methods Semen samples were collected from 18 infertile men with spinal cord injury (SCI) by penile vibratory stimulation (PVS) and from 14 psychogenic anejaculation (PA) patients by percutaneous vasal sperm aspiration (PVSA). These semen samples as well as samples from 16 donors were analyzed using the hypo-osmotic swelling (HOS) test, the sperm chromatin dispersion (SCD) test and multi-color fluorescence in situ hybridization (FISH) with probes specific for chromosomes 13, 18, 21, X and Y.

Results There were significant differences in the percentages of motile sperm, normal morphologic sperm and sperm DNA fragmentation between the infertile men with SCI and the control group ($P<0.05$ and $P<0.01$). The sperm motility was significantly greater in the PA-PVSA group than in the SCI-PVS group ($P<0.01$). The number of round cells per mL of semen obtained from the 18 SCI patients by PVS was between 1 and 8 million. The rate of sperm DNA fragmentation in the SCI-PVS group was higher than that of the PA-PVSA group ($P<0.05$). The aneuploidy rates for the

SCI patients were 2.4-fold higher for chromosomes 13, 18 and 21 and 2.2-fold higher for chromosomes X and Y than for patients in the control group ($P<0.0001$).

Conclusions The semen quality is poorer, sperm DNA fragmentation and sperm chromosomal aneuploidies are seen at a higher rate for SCI patients compared to healthy, fertile and normospermic men. Whether the difference in yield is due to increased scrotal temperature, genitourinary infection, or other reasons requires further study.

Keywords Sperm chromatin dispersion (SCD) test · FISH · Sperm chromosomal aneuploidy · Anejaculation · Male infertility

Introduction

Anejaculation is one of the sexual disorders in men and commonly diagnosed on the basis of history and lack of sperm in urine specimens. The reasons of anejaculation are primarily caused by spinal cord injury (SCI) or psychological factors [1–4]. Numerous studies have shown that there are about 85% of spinal cord-injured men permanently lose their ability to ejaculate [3–5]. Psychogenic anejaculation (PA) is one of the ejaculatory disorders. Men who suffer from PA are otherwise healthy individuals who cannot consciously ejaculate even by masturbation, although they may have erections and nocturnal emissions. Numerous treatments for anejaculation to obtain semen are available and include psychotherapy, oral treatment with phosphodiesterase-5 inhibitors, intracavernosal injection or intraurethral suppositories of alprostadil, vacuum devices, penile implants, rectal probe electroejaculation (EEJ) and penile vibratory stimulation (PVS) [6–11]. However, some patients can not ejaculate to obtain the semen sample. The PVS [8, 12] and EEJ [13, 14] have been established for obtaining semen in these anejaculatory men. The success rates

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Capsule The rate of sperm chromosomal aneuploidy and DNA integrity for anejaculatory patients with spinal cord injury was significantly higher than healthy, fertile and normospermic men.

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for recovering spermatozoa by high-amplitude PVS have been reported to be 80% [2], and by EEJ have been consistently 70–90% [1, 15, 16]. However, the EEJ method has been reported to obtain poor-quality semen (asthenozoospermia and teratozoospermia) from SCI patients [7, 15, 17]. For the collection of semen from infertile men experiencing anejaculation caused by PA, the percutaneous vasal sperm aspiration (PVSA) method was established by Qiu et al. (2003) [4].

Sperm morphology, motility, and concentration are the 3 most important factors for male reproduction potential [18]. However, these parameters are not sufficient to assess every aspect of sperm function and quality. Therefore, more sensitive diagnostic techniques to evaluate sperm function and quality must be developed. The hypo-osmotic swelling (HOS) test can evaluate sperm motility [19, 20] and can detect the integrity of the tail membrane of sperm. In recent years, it has been reported that sperm DNA fragmentation can be evaluated in a variety of ways, including the sperm chromatin structure assay (SCSA) [21, 22], terminal deoxynucleotidyl transferase-mediated d UDP nick-end labelling (TUNEL) [23–25], single cell gel electrophoresis (COMET) assay [26], acridine orange staining technique (AOT) [27] and the sperm chromatin dispersion (SCD) test [28–30]. The TUNEL, AOT, and SCD are all simple, less expensive procedures and can be performed in a short period of time [29]. However, the vitality and the integrity of the sperm membrane could not be determined using these tests.

Cytogenetic analyses of sperm nuclei using the fluorescence in situ hybridization (FISH) technique with different chromosome-specific probes are one preferred method for the evaluation of sperm chromosomal aneuploidy [31]. FISH has been used to study numerical chromosomal abnormalities in human sperm nuclei from infertile and fertile men. Findings [32, 33] have demonstrated a high degree of asthenoteratozoospermia and abnormal chromatin condensation reduced binding of a fluorescent acrosomal marker in men with SCI. Brackett et al. [34] reported that the DNA fragmentation index (DFI) is seen at a higher rate compared to healthy, fertile and normospermic men.

In this study, semen samples were collected from 18 SCI patients using PVS or from 16 PA patients using PVSA. Sperm membrane and DNA integrity were assessed using HOS and HOS/SCD test, and sperm chromosomal aneuploidy was analyzed using the FISH technique.

Materials and methods

Patients

Between February 2006 and February 2011, semen samples were obtained from male partners of infertile couples ($n=34$) who had visited the Fertility Center of Shandong Research

Institute for Family Planning for evaluation and infertility treatment. Semen samples were also collected from fertile volunteer sperm donors ($n=16$). For the 18 patients, the mean duration of time ($\pm$ SD) since the SCI was 8.2 ± 3.2 years (range, 3–11 years). Psychogenic anejaculation (PA) was diagnosed on the basis of lack of physical causes for the problem, presence of occasional nocturnal emissions and sexual relations without conscious ejaculations ($n=16$). The levels of thyroid-stimulating hormone, FSH, LH and prolactin were within the normal range in the 16 PA patients. All of the volunteer donors had previously fathered at least one child. There was no significant difference between the mean age ($\pm$ SD) of the anejaculatory patients (33.2 ± 2.6 years; range, 27–36 years, $n=34$) and the donors (mean age $\pm$ SD: 32.9 ± 2.1 years). The mean duration of time ($\pm$ SD) since the onset of infertility was 6.8 ± 4.1 years for the 34 patients (range, 1.5–11 years). Prior to this study, patients were informed of the investigations and provided consent. This study was reviewed by the ethics committee of Shandong Provincial Institute of Science and Technology for Family Planning in China. Each of the 34 patients had requested infertility treatment and had provided semen samples for intrauterine insemination (IUI) or in vitro fertilization (IVF). The identification information of each of the subjects in this study was kept confidential and was protected from the public.

Semen collection and analysis

Semen samples from the 16 healthy donors (control group) were collected by masturbation and ejaculation into sterile glass cups after 3 to 5 days of abstinence, and the samples were analyzed on-site for both macroscopic and microscopic characteristics within 1 h of collection [19, 20]. For semen retrieval, each of the SCI patients and the PA patients was first questioned regarding his ability to obtain semen by masturbation. For cases where the patient was unable to ejaculate, the prostatic massage was attempted. If this failed, PVS was attempted using a WAHL 2-speed massager for 18 SCI patients (SCI-PVS group) (Model 4160-Type, USA). If the PVS procedure was also unsuccessful, PVSA was performed (sperm was obtained from 16 PA patients [PA-PVSA group] using this method). The PVSA procedure has been described elsewhere by Qiu et al. [4, 35]. Sixteen procedures were thoroughly described to all subjects ($n=16$), including semen retrieval methods and intracorporeal injection. Written consent was provided by the subject before any procedure. Local anesthesia (2% lidocaine) of scrotum and spermatic cord was administered for the patients. Sperm procurement was successful without any complications in 16 patients. Following the liquefaction or PVSA procedures, a standard semen analysis was performed for all of the obtained samples, according to the criteria of the World Health Organization (WHO) [19], for the

determination of semen volume, sperm concentration, motility (motile sperm) and morphology. Sperm morphology was assessed using the strict WHO criteria after Diff-Quik staining [20]. Information regarding the semen source (patients or fertile volunteers) was withheld from the technologists who performed the semen analyses, the FISH and the DNA fragmentation testing.

The HOS/SCD test

The HOS test was performed as recommended by the WHO [19, 20]. For the preparation of the HOS solution, 0.735 g sodium citrate dihydrate (S4641, Sigma USA) and 1.351 g d-fructose (F3510, Sigma USA) were dissolved in 100 mL purified water. A total of 0.1 mL of the semen sample was mixed with 1.0 mL of a hypo-osmotic solution (150 mOsm). After incubation for 5 min at 37°C, ≥ 200 spermatozoa were analyzed using a phase-contrast microscope (Olympus, IX51, Japan) at 400 $\times$ magnification. The modifications of the sperm tails were evaluated, and the scores for swollen sperm and unswollen sperm were reported as the percentage of the total sperm observed. Sperm tail swelling was characterized as either “tail membrane-intact” or “normal HOS”. In contrast, unswollen sperm were classified as “abnormal HOS”.

The SCD test was performed according to the methods published by Fernández et al. [28] and Zhang et al. [30]. After the HOS test had been performed, the SCD test was immediately carried out to evaluate the sperm DNA fragmentation of the 34 patients and the 16 donors. One mL of the semen sample (mixed with hypo-osmotic medium) from the HOS-test was centrifuged at 500g for 5 min, and then the supernatant was discarded and the sperm pellet was resuspended in 0.1 mL of a hypo-osmotic solution. The suspension was incubated for 5 min at 37°C, and ≥ 200 spermatozoa were analyzed using a phase-contrast microscopy (Olympus, IX51, Japan). Modifications of the sperm tails were evaluated, and the number of swollen sperm was assessed. The sperm sediment was resuspended and diluted to 10 million/mL with PBS (pH 6.8). The suspensions were mixed with a 1% low-melting point aqueous agarose at 37°C (to obtain a 0.7% final agarose concentration). Aliquots containing 50 μ L of this mixture were pipetted onto a glass slide precoated with 0.65% standard agarose. The slides were then dried at 80°C, covered with a coverslip (22 $\times$ 22 mm) and were left to solidify for 5 min at 4°C. The coverslips were carefully removed, and the slides were immediately immersed horizontally into a tray containing a freshly prepared acid denaturation solution (0.08 N HCl). The slides were immersed for 7 min at 22°C in the dark to generate restricted single-stranded DNA (ssDNA) motifs from DNA breaks. The denaturation was then halted, and the proteins were removed by transferring the slides to a tray containing a neutralizing and lysing solution (0.4 M Tris, 0.8 M DTT, 1% Triton-100 and

50 mM EDTA, pH 7.3), where they were kept for 20 min at room temperature. The slides were thoroughly washed in Tris-borate-EDTA (Sigma Inc., USA) buffer (0.09 M Tris-borate and 0.002 M EDTA, pH 7.5) for 3 min, dehydrated in sequential 70%, 90%, and 100% ethanol baths (2 min each) and were then air-dried. The cells were then stained with DAPI (4,6-diamidino-2-phenylindole) (2 mg/mL) (Vysis, USA) for bright-field microscopy, which involved staining for 10 min, washing with distilled water and air-drying.

Scoring A total of 500 spermatozoa were evaluated manually on each slide under bright-field optics and oil immersion at 1,000 $\times$ magnification (Leica, DM4000B, Germany). The patterns observed for the HOS/SCD test were classified as follows:

- (1) pattern 1: spermatozoa with large DNA dispersion halos or medium-sized halos and swollen tails (DNA integrity and membrane-intact);
- (2) pattern 2: spermatozoa with large DNA dispersion halos or medium-sized halos and unswollen tails (DNA integrity and membrane-damaged);
- (3) pattern 3: spermatozoa with small-sized halos or the absence of a halo and swollen tails (DNA integrity-damaged and membrane-intact);
- (4) pattern 4: spermatozoa with small-sized halos or the absence of a halo and unswollen tails (DNA integrity-damaged and membrane-damaged).

The cells were evaluated for swollen tails, halo size and dispersion pattern. The criteria for large-, medium- and small-sized sperm halos were based on the standards of Fernández et al. [28] and Zhang et al. [30]. The nuclei with large- to medium-sized halos were considered to represent sperm with non-fragmented DNA, whereas nuclei with a small-sized halo or those that were degraded and without a halo were considered to represent sperm with fragmented DNA.

FISH analysis

To evaluate the aneuploidy frequency, FISH was performed as described by Hristova et al. [36]. The spermatozoa were diluted in PBS and washed three times by centrifugation for 10 min at 750g. Then resuspended the pellet in fresh fixative and added 500 μ L dithiothreitol (DTT, Sigma Inc., USA) into the sperm fluid to enlarge the sperm head. The spermatozoa suspension was spread onto clean glass slides and air-dried, then stored the slides at -20°C for further use.

The slides were dehydrated through ethanol and dried at room temperature and placed at 73°C for 4–5 min. The probe mixture contained the probes specific for chromosomes X, Y, and 18, and 13 and 21. Ten μ L probe mixtures

Table 1 Comparison of semen parameters (mean $\pm$ SD) between PA-PVSA, SCI-PVS and control group

Subjects	<i>n</i>	Semen volume (ml)	Concentration ($\times 10^6$ /ml)	Motility (%)	Normal morphology (%)
Control	16	2.8 $\pm$ 0.6	79.0 $\pm$ 20.9	78.2 $\pm$ 8.6	32.1 $\pm$ 6.8
PA-PVSA	16		49.5 $\pm$ 21.6 ^a	75.8 $\pm$ 7.1	28.0 $\pm$ 8.8
SCI-PVS	18	2.6 $\pm$ 0.7	82.6 $\pm$ 23.1 ^b	28.2 $\pm$ 10.9 ^{a b}	10.9 $\pm$ 7.6 ^{a b}

PA-PVSA: sperm retrieved by percutaneous vasal sperm aspiration in psychogenic anejaculation men. SCI-PVS: sperm retrieved by penile vibratory stimulation in men with spinal cord injury. ^a $P < 0.01$, compared to control, ^b $P < 0.01$, compared to PA-PVSA group

were added to the slides and placed into dark box at 37°C over night for hybridization.

Three-color and dual-color FISH experiments were performed using chromosome enumeration DNA probes (CEP, Vysis, USA) for the X chromosome (DXZ1 Locus, Spectrum-Green), the Y chromosome (DYZ3 locus, Spectrum-Orange), and for chromosome 18 (D18Z1 Locus, Spectrum-aqua). These experiments also used locus-specific identifier DNA probes (LSI, Vysis, USA) for chromosomes 13 (D13Z2 Probe-Green) and 21 (D21Z1 Probe-Orange). The FISH assay was performed according to the protocol recommended by the manufacturer. Preparations were counterstained with a DAPI (Vysis Inc., USA). Slides were screened using a 100 $\times$ objective on a fluorescence microscope (Nikon E1000, Japan) equipped with a DAPI triple band-pass filter and excitation of 450–490 nm. Cover-slips were placed over the hybridization area, sealed with rubber cement.

Scoring of sperm nuclei The overall hybridization efficiency was >99%. The image analysis system consisted of the FISH analysis software (British Applied Image UK). No slide was scored unless the hybridization rate was greater than 95%. More than 5,000 spermatozoa were analyzed per sample, and split signals were not scored as disomies. Spermatozoa were scored as nullisomic if they showed no signal for a given chromosome, provided that the signal of the other chromosome tested was present. Finally, a spermatozoon was considered diploid when it manifested two signals for each tested chromosome and when the tail as well as the normal oval shape of the sperm head was evident. The absence of any FISH signals in a spermatozoon head that

had also showed DAPI staining was considered to represent a lack of hybridization.

The statistical analysis

All data were analyzed using the SPSS 13.0 package software (SPSS Inc, Chicago). Statistical significance was evaluated using the Student's *t*-test. Analysis of variance and the post hoc test were used for comparison between more than two groups. To compare differences in the aneuploidy of chromosomes 13, 18, 21, X and Y between SCI patients, PA patients and controls, a χ^2 test was used. The difference was considered statistically significant for *P* values <0.05.

Results

The results of the semen analysis from the 34 patients and the 16 donors

The basic seminal parameters of the male patients and the fertile volunteers are summarized in Table 1. For all of the 18 patients with SCI, sexual intercourse and self-ejaculation were unsuccessful. Prior to performing PVSA, 4 of the 16 patients with PA had been able to obtain semen using the PVS method during the previous month. However, when the wives of these patients entered the IUI or IVF treatment cycle, the semen was unable to be collected via prostatic massage or PVS. As a result, these patients agreed to the PVSA procedure to obtain sperm for the IUI or IVF

Fig. 1 The sperm morphology and round cells of the control and SCI-PVS group after Diff-Quik staining. **a** Control group. **b** SCI-PVS group, red arrow shows round cell. Bright-field optics at 1,000 $\times$ magnification and oil immersion

treatment. For statistical analysis, these 4 PVS samples were not included in the statistics. Spermatozoa were successfully collected from the 16 PA patients by PVSA for the IUI or IVF treatment cycles. Semen samples were obtained from the 16 fertile donors by masturbation and from 18 SCI patients by PVS. The spermatozoa concentration ranges for the control group, the PA-PVSA group and the SCI-PVS group were from $58 \times 10^6/\text{mL}$ to $105 \times 10^6/\text{mL}$, $9 \times 10^6/\text{mL}$ to $88 \times 10^6/\text{mL}$ and $10 \times 10^6/\text{mL}$ to $115 \times 10^6/\text{mL}$, respectively. The sperm motility was significantly greater for the PA-PVSA group and the control group than for the SCI-PVS group ($P < 0.01$), although the sperm concentration in the PVSA group was significantly lower than that of the SCI-PVS group and the control group ($P < 0.01$). The percentages of the normal morphology for the PA-PVSA group were similar to the control group ($P > 0.05$) and higher than that of the SCI-PVS group ($P < 0.01$). In the PA-PVSA group, 0.05–0.15 mL semen from the vas deferens and epididymis was aspirated into the 1.0 mL of sperm preparation medium, therefore, the semen volume did not compare to the control. None of the semen samples from the 16 donors or the 16 infertile patients with PA whose semen obtained by PVSA had significant leukocytospermia according to WHO guidelines (< 1 million round cells per mL), although the number of cells per mL of the semen samples obtained by PVS from 18 SCI patients was between 1 and 8 million. The semen smears of the control group and the SCI-PVS group after Diff-Quik staining are shown in Fig. 1.

The results of the HOS/SCD test for the fertile donors and the anejaculatory patients

The average percentages of normal HOS, abnormal HOS and DNA fragmentation from the HOS test and the SCD test, and samples demonstrating pattern 1, pattern 2, pattern 3 and pattern 4 from the HOS/SCD test for the control group are shown in Table 2. The sum rates of pattern 1 plus pattern 2 were calculated as 86.7% ($75.2\% + 11.5\%$). No DNA fragmentation was observed in these spermatozoa. The sum rates of pattern 1 plus pattern 3 were 76.9% ($75.2\% + 1.7\%$). The average percentage of sperm with large or medium-sized halos from the SCD test alone was significantly higher than that of the sperm with normal HOS from the HOS test (86.7% versus 77.6% , $P < 0.05$), in contrast, the average percentage (13.8%) of spermatozoa with small halo or absence halo from the SCD test alone was significantly lower than that of abnormal HOS (22.4%) from the HOS test alone ($P < 0.01$). No significant difference was observed in the percentages of sperm with small halo and absence halo between the HOS/SCD test (pattern 3 plus pattern 4, $1.7\% + 11.6\% = 13.3\%$) and the SCD test alone (13.3% versus 13.8% , $P > 0.05$) (Table 2).

Table 2 The percentages (mean $\pm$ SD) of normal HOS, abnormal HOS and fragmented DNA from the HOS, the SCD and the HOS/SCD test for the 16 fertile men (control)

HOS test (%)		SCD test (%)		HOS/SCD test (%)	
Normal HOS	77.6 $\pm$ 5.8	Normal SCD	86.2 $\pm$ 7.5 ^a	Pattern 1	75.2 $\pm$ 6.7
				Pattern 2	11.5 $\pm$ 1.5
Abnormal HOS	22.4 $\pm$ 2.1	Abnormal SCD	13.8 $\pm$ 2.7 ^b	Pattern 3	1.7 $\pm$ 0.6
				Pattern 4	11.6 $\pm$ 1.8

Normal SCD: spermatozoa with large halos or medium-sized halos. Abnormal SCD: spermatozoa with small halos or without halo. Normal HOS: swollen tail. Abnormal HOS: unswollen tail. Pattern 1: spermatozoa with large DNA dispersion halos or medium-sized halos and swollen tails. Pattern 2: spermatozoa with large halos or medium-sized halos and unswollen tails. Pattern 3: spermatozoa with small halos or absence halo and swollen tails. Pattern 4: spermatozoa with small halos or absence halo and unswollen tails

^a $P < 0.05$ and ^b $P < 0.01$, compared with the HOS test alone

The average percentages of pattern 1, pattern 2, pattern 3 and pattern 4 from the HOS/SCD test for the control group, the PA-PVSA group and the SCI-PVS group are shown in Table 3. No significant difference was found in the percentages of pattern 1, 2, 3 and 4 between the PA-PVSA group and the control group. There were significantly fewer samples with a pattern 1 result identified from the SCI-PVS group compared to the control group ($P < 0.01$). In contrast, the percentage of samples displaying pattern 2 and 4 was significantly higher in the SCI-PVS group than in the control group ($P < 0.05$ and $P < 0.01$) and the PA-PVSA group ($P < 0.01$). Smears from the HOS/SCD test are shown in Fig. 2.

Sperm aneuploidy rate

The numerical chromosome abnormalities and comparison among the 3 groups are shown in Tables 4 and 5. By Chi-square analysis, the differences of numerical chromosome abnormality rate of chromosomes 13, 18, 21, X, and Y in the SCI-PVS group were statistically significant, compared to the control group ($P < 0.0001$). There were no significant

Table 3 Comparison of the percentages (mean $\pm$ SD) of spermatozoa with swollen tails and fragmented DNA by HOS/SCD test between the PA, the SCI and the control group

	<i>n</i>	Pattern 1	Pattern 2	Pattern 3	Pattern 4
Control	16	75.2 $\pm$ 6.7	11.5 $\pm$ 1.5	1.7 $\pm$ 0.6	11.6 $\pm$ 1.8
PA-PVSA	16	74.8 $\pm$ 3.1	13.2 $\pm$ 1.2	1.1 $\pm$ 0.8	10.9 $\pm$ 2.2
SCI-PVS	18	35.3 $\pm$ 3.7 ^{b, c}	16.0 $\pm$ 2.1 ^a	3.2 $\pm$ 1.9	45.5 $\pm$ 5.6 ^{b, c}

^a $P < 0.05$ and ^b $P < 0.01$, compared with control, ^c $P < 0.01$, compared with PA

Fig. 2 HOS and SCD test. **a** SCD test alone, sperm with large or medium-sized halos. **b** The HOS/SCD test. N, no halo in sperm head; S, small halo (pattern 4); L, large halos and swollen tails (pattern 1). **c** Pattern 1. **d** Pattern 2. **e** Pattern 3. **f** Pattern 4. **g** Swollen tail and no halo in sperm head. **h** No halo in sperm heads (double heads) and unswollen tail. Bright-field optics at 1,000 × magnification and oil immersion

differences in the sum of aneuploidy number for chromosomes 13, 18 and 21 or the sum of aneuploidy number for chromosomes X and Y between the PA-PVSA group and the control group ($\chi^2=0.774$, $P=0.379$ and $\chi^2=0.068$, $P=0.795$). In the SCI-PVS group, the sum rates of chromosomal aneuploidy for chromosomes 13, 18, and 21 were 2.4-fold (3.09%/1.31%) times greater than those for the control group ($P<0.001$), and the sum rates of aneuploidy for chromosomes X and Y in SCI-PVS group were 2.2-fold (1.65%/0.74%) times greater than those for the control group ($P<0.001$). Figures 3 and 4 show images of the sperm FISH in the control group and the SCI-PVS group.

Discussion

In present study, the high quality spermatozoa were collected by the PVSA technique in 16 infertile patients with PA. The percentage of sperm motility and normal morphology in these PA patients was similar to the donors. It suggests that the spermatogenesis in most infertile patients caused by PA may be normal. PVSA is a minimally invasive procedure that can be performed under local anesthesia. In this procedure, the spermatozoa are directly aspirated from the vas deferens into the sperm preparation medium. Then, via IUI or IVF, a pregnancy can be established for infertile couples

Table 4 Aneuploid number of chromosomes 13, 18, 21, X and Y in the SCI-PVS, the PA-PVSA group and the control group

Group	13	18	21	X	Y	
Control (<i>n</i> =16)	281 (0.39)	324 (0.45)	338 (0.47)	288 (0.40)	245 (0.34)	
PA-PVSA (<i>n</i> =16)	410 (0.44)	406 (0.44)	445 (0.48)	390 (0.42)	245 (0.33)	
SCI-PVS (<i>n</i> =18)	950 (0.96)	1000 (1.01)	1109 (1.12)	901 (0.91)	733 (0.74)	
Control: PA-PVSA	χ^2	2.579	0.144	0.087	0.406	0.135
	P	0.108	0.705	0.768	0.524	0.713
Control: SCI-PVS	χ^2	189.042	170.211	210.392	157.083	117.358
	P	0.001	0.001	0.001	0.001	0.001

Table 5 Comparison of the sum of aneuploidies in chromosomes 13, 18 and 21 and in chromosomes X and Y between the PA, the SCI and the control group

Subjects	<i>n</i>	Total sperm number	Aneuploidy 13/18/21 (%)	Total sperm number	Aneuploidy X/Y (%)
Control	16	72000	943 (1.31)	72000	532 (0.74)
PA-PVSA	16	92800	1262 (1.36)	92800	696 (0.75)
SCI-PVS	18	99000	3059 (3.09)	99000	1633 (1.65)
χ^2	Control :	SCI	577.965		276.506
χ^2	PA :	SCI	650.998		323.085
<i>P</i>			<0.0001		<0.0001

whose infertility is caused by male factors, such as azoospermia due to reproductive tract obstruction, retrograde ejaculation or anejaculation [4]. In this study, semen samples were successfully obtained by PVS from 18 SCI patients, and the rates of sperm motility, normal morphology and normal HOS/SCD in these patients were significantly lower than controls. Hovav et al. [13] reported that the concentration and the motility of the spermatozoa obtained using electroejaculation from 9 men who suffered from psychogenic anejaculation were not significantly different from sperm obtained naturally. The semen quality of men with SCI is poor, and these changes can be seen as early as 2 weeks after injury [10]. The distinguishing characteristics of poor-quality sperm are abnormal motility and viability. In the majority of the men with SCI, the sperm concentration can be normal, but sperm motility, viability and normal morphology are typically lacking. In our study, only one of 18 semen samples obtained by PVS from patients with SCI (1/18) shown oligozoospermia ($10 \times 10^6/\text{mL}$), and others were in normal range.

There are reliable methods to induce release of semen in SCI men, including stimulation of a reflex ejaculation by penile vibration and electrical stimulation of seminal emission by rectal probe electroejaculation (EEJ). EEJ may also be used for men who have neurogenic anejaculation due to retroperitoneal surgery, diabetic neuropathy, multiple sclerosis and other conditions which affect this reflex. Sperm

from induced ejaculation can be harvested and used for artificial insemination, IUI [4, 37], IVF [38] or intracytoplasmic sperm injection [37, 39].

The PVS procedure less often gives retrograde or partly retrograde emission and the antegrade semen specimens often have a better volume and are not contaminated with urine. This technique should therefore give a better quality of semen. Moreover, the vibrator is easy to handle, even for persons with impaired hand function, which makes it suitable for domestic use [40]. Spermatozoa obtained by PVSA were directly aspirated into the sperm preparation medium and were not mixed with seminal vesicle fluid and prostatic fluid, which resulted in the greater sperm forward motility of the PVSA group than the PVS group. The poor-quality sperm obtained from spinal cord-injured men may be related to an increased scrotal temperature, urinary infections, a stasis of seminal fluid, neural effects on the physiology of the testis and the epididymis, sperm autoimmunity or a reduced blood flow to the testis. The spinal cords are not injured for patients with PA, therefore the semen quality may be similar to the fertile donors.

The WHO recommends the hypo-osmotic swelling (HOS) test and eosin Y staining to be used as the basic methods for testing sperm function. These methods assess the vitality of sperm based on the assumption that cells with intact membranes (live cells) will swell in hypotonic conditions and will not incorporate eosin Y staining. The

Fig. 3 Sperm FISH with chromosome X centromere (green), chromosome Y centromere (red) and chromosome 18 centromere (blue) specific probes. **a** Control group, sperm with one chromosome X and one chromosome 18, or sperm with one chromosome Y and one chromosome 18. **b** SCI-PVS group, yellow arrow shows a spermatozoon with one chromosome Y and two chromosomes 18, and red arrow shows a

spermatozoon with one chromosome X, one chromosome Y and one chromosome 18. **c** SCI-PVS group, yellow arrow shows a spermatozoon with one chromosome X and without chromosome 18, and red arrow shows a spermatozoon with two chromosomes X and two chromosomes 18. Fluorescence microscope at $1,000 \times$ magnification and oil immersion

Fig. 4 Sperm FISH with chromosome 13 centromere (green) and chromosome 21 centromere (red) specific probes. **d** Control group, sperm with one chromosome 13 and one chromosome 21. **e** SCI-PVS group, yellow arrow shows a spermatozoon with one chromosome 21 and without chromosome 13, and red arrow shows a spermatozoon

with one chromosome 21 and two chromosomes 13. **f** SCI-PVS group, yellow arrow shows a spermatozoon with two chromosomes 21 and one chromosome 13. Fluorescence microscope at 1,000 × magnification and oil immersion

percentage of live spermatozoa is assessed by identifying those with an intact cell membrane by hypotonic swelling (WHO, 1999) [19, 20]. The HOS test presumes that only cells with intact membranes (live cells) will swell in hypotonic solutions (WHO, 1999) [19, 20]. Researchers have reported that HOS provides the simplest and least expensive measurement of sperm tail membrane integrity and vitality based on a number of different tests of sperm function [41]. The osmotic stress caused by the chosen hypo-osmotic medium must be sufficient to affect an influx of water into the cell to result in an increase in volume and hence curling of the tail, but to prevent lysis of the sperm membrane [42, 43]. Furthermore, an HOS score less than 50% is rarely associated with pregnancy in vivo [43, 44]. Yet, other researchers consider the HOS score alone to not be sufficiently useful for predicting fertilization rates [45, 46].

The sperm DNA fragmentation index is an important parameter used to assess sperm quality. The sperm chromatin dispersion (SCD) test is a simple and reliable procedure that does not require elaborate or expensive methodologies [28]. Sperm DNA fragmentation may result from aberrant chromatin packaging during spermatogenesis, defective apoptosis prior to ejaculation or oxidative stress. A high degree of DNA fragmentation is determined in undiagnosed/unexplained infertile males [47]. Thus, the determination of DNA fragmentation in human sperm is important. Sperm DNA fragmentation and membrane integrity may be simultaneously determined for the same spermatozoon by performing the SCD test immediately after the HOS test.

In our results, the sum rates of pattern 1 plus pattern 2 (75.2%+11.5%) for 16 fertile donors were similar to the percentage of nuclei with large DNA dispersion halos or medium-sized halos (86.2%) using SCD test alone. We did not observe statistically significant difference between the sum rates of pattern 1 plus pattern 3 (75.2%+1.7%) and the percentage of swelling sperm (77.6%) using the HOS test alone (tail membrane-intact). The sum rates of pattern 3 and pattern 4 (1.7%+11.6%) using the HOS/SCD test was similar to the rates of sperm with small halo or absence halo

(13.8%) using SCD test alone ($P>0.05$). These indicate that the results of sperm DNA fragmentation using the HOS/SCD test could not be interfered by the prior HOS test. The HOS/SCD test may be served as a useful method for determining the integrity of the tail membrane and DNA in the same spermatozoon simultaneously. Spermatozoa with swollen tails (normal HOS) and those with small halos or nonexistent halos may be dead or chromatin-damaged, whereas spermatozoa with unswollen tails (abnormal HOS) and those with large halos or medium halos may be alive and chromatin-intact.

In the present study, There were significantly fewer samples with a pattern 1 result identified from the SCI-PVS group compared to the control group ($P<0.01$). In contrast, the percentage of samples displaying pattern 2 and 4 was significantly higher in the SCI-PVS group than in the control group ($P<0.05$ and $P<0.01$) and the PA-PVSA group ($P<0.01$). The rate of numerical chromosomal abnormalities for chromosomes 13, 18, 21, X and Y was higher for samples from the SCI group compared to the control group ($p<0.001$). For SCI patients, it seems reasonable to assume that neurotrois and malnutrition of the lower limbs together with the prolonged storage of sperm due to anejaculation may have caused a gradual loss of sperm motility and an increase in sperm DNA fragmentation and numerical chromosomal abnormalities. The poor-quality sperm obtained from spinal cord-injured men may be related to an increased scrotal temperature, urinary infections, a stasis of seminal fluid, neural effects on the physiology of the testis and the epididymis, sperm autoimmunity or a reduced blood flow to the testis [17]. Our results show that the number of round cells per mL of the semen samples obtained by PVS from 18 SCI patients was between 1 and 8 million. The genitourinary infections were obviously present in the SCI patients, and it may be one of the important reasons of impacting semen quality.

Recently, the integrity of mammalian sperm DNA and the sex chromosomes has become a focus of research, as this integrity is considered to be of prime importance for the paternal genetic contribution of healthy offspring. Chromosome

aneuploidy and DNA decondensation or damage are correlated with reproductive failure. Damaged DNA and sperm chromosomal aberrations can have a significant negative impact on oocyte fertilization, the rate of embryo development and the live-birth rate. A significant correlation between the presence of nuclear DNA alterations in mature spermatozoa and poor sperm parameters or impaired reproductive efficiency, and a positive relationship between spermatozoa with total sperm head birefringence in their head and increased DNA fragmentation are reported in both humans and animals [48–52]. In the human ejaculate, there is a very heterogeneous sperm population, with various degrees of nuclear maturity. Hristova et al. [37] performed sperm chromosome FISH analysis for oligoasthenozoospermia infertile patients and found the ratio of sperm chromosomal aberration was approximately 2- to 10-fold higher in these patients than in fertile males. Our results suggest that infertile patients with SCI have a greater frequency of numerical chromosome abnormalities (2.4-fold for chromosomes 13, 18 and 21; 2.2-fold for chromosomes X and Y). There was no significant difference between the numerical chromosome abnormalities of chromosomes 13, 18, 21, X and Y of the PA-PVSA group and the control group.

Ejaculatory dysfunction and poor quality semen are two main causes for the impaired reproductive potential in SCI men. The semen from men with SCI has commonly been characterized by small volume, low count and low motility [53, 54]. Insufficient drainage, genitourinary infections and raised scrotal temperature are thought to cause the impairment [53]. Perkash et al. [55] reported that testicular dysfunction is common in SCI men. Testicular biopsies disclose varying degrees of tubule degeneration and decreased spermatogenic activity [55]. In addition, these SCI men could not self-ejaculate, the retention time of spermatozoa in the epididymis and the vas deferens was prolonged. Therefore, these factors may have negatively impacted the quality of sperm in these individuals.

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